RESULTS OF MOLECULAR TESTING OF SAMPLES FROM PAKISTAN

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ABSTRACT

Introduction
FMD is endemic in Pakistan and causes significant economic losses in livestock farming, in particular in dairy buffalo and cattle. In an accompanying paper we describe the sampling, shipping and real-time RT-PCR results of samples collected at the Landhi Dairy/Cattle Colony (LCC) outside Karachi in Pakistan and in the present paper we describe the results of the sequence analysis performed on the same samples.

Materials and methods
From April 2006 to April 2007 we collected mouth swabs and epithelial samples from a large number of animals in LCC. Samples were shipped to Lindholm, Denmark, as either stabilised and inactivated swab samples in RNA stabilising buffer (i.e. Qiagen RLT buffer), as cDNA prepared at the National Reference Laboratory in Islamabad, Pakistan, or in a few cases as vesicular epithelium containing live virus and shipped as dangerous goods via the WRL at Pirbright Laboratory, UK (kindly assisted by David Paton, Nigel Ferris and Geoff Hutchings). PCR-based sequencing of the VP1 and capsid region as well as full genome sequencing was performed using standard techniques.

Results
The results of the sequencing and phylogenetic analyses are presented. A high number of samples contained serotype O of the Pan Asia II lineage or serotype A of the A Iran 2005 lineage.

Discussion
Sequencing of FMDV RNA present in mouth swabs rendered stable and non-infectious by a suitable stabilising buffer provides a valuable and useful alternative to sequencing based on infectious epithelial samples and provides reliable sequence information for characterisation of circulating strains of FMDV in an endemic situation as observed in the LCC in Pakistan. Preliminary studies indicate (data not shown) that epithelial samples may also be stabilised and rendered non-infectious “on site“ by using e.g. Ambion RNAlater, and consequently, shipment of samples containing infectious FMDV, which is both cumbersome, expensive and may potentially cause infection if accidentally released, appears no longer to be required for prevalence studies and molecular analysis including sequencing and strain characterisation. Moreover, as samples shipped in a stabilised form may after RNA extraction be used for re-generating infectious virus by transfection into susceptible cell cultures (Graham Belsham, personal communication), this approach may truly provide a significant leap forward for a thorough knowledge-based vaccination approach for FMD control in difficult endemic settings.

INTRODUCTION
The Landhi Dairy/Cattle Colony (LCC) is located in the eastern suburbs of Karachi and is the largest dairy colony in Pakistan and the biggest buffalo colony worldwide. There is estimated to be a total
of around 250-280 000 buffalo and 15-25 000 cattle in the colony and that there are between 1000-2000 farms each with around 100-200 animals on average. All of this is located on only 750 acres of land (including 250 acres for primitive roads).

Additional background information regarding the LCC, the collaborative project involved, the collection and shipping of samples and the results of real-time RT-PCR analysis is described in an accompanying paper.

2. MATERIALS AND METHODS

2.1 Virus isolation and sequencing.

The study combines both field and molecular epidemiology and was planned as a longitudinal and cross-sectional survey in the Landhi Dairy/Cattle Colony (LCC). Samples were collected during field trips to Pakistan in January/February, April and September 2006 and January and April 2007. In addition, a smaller number of samples were collected by local Veterinary Officers each month. Mouth swab samples were collected from randomly selected animals while epithelial samples were collected from animals showing clinical signs of acute FMD. A few epithelial samples were also collected from a small farm just outside LCC and from the Nagori Cattle Colony, a smaller dairy colony about 50 km from Karachi with around 10 000 animals (mainly buffalo) and with less crowded conditions for the animals and with better animal husbandry than LCC. The location of all farms being sampled was logged by handheld GPS equipment and the farmers were thoroughly questioned for knowledge that may be of relevance for the analysis. Epithelial samples were collected using standard procedures in phosphate-buffered 50% glycerol and these samples were stored at –20°C until shipped on dry ice. Epithelial samples were initially sent to the World Reference Laboratory (WRL) at Pirbright, UK, to allow sharing of samples and results. From October 2006 to March 2007 we collected monthly serum samples from 30 randomly selected buffaloes immediately after slaughter at the local slaughterhouse in LCC. Virus isolation, ELISA, real-time RT-PCR, sequencing and phylogenetic analysis were done using standard methods as described in the accompanying paper and in previous publications 3, 4.

For additional details including how mouth swab sampling was organized and handled please see the accompanying paper.

3. RESULTS

3.1 Infection prevalence

The prevalence of samples positive for FMDV RNA by real-time RT-PCR analysis of mouth swab samples collected from randomly selected animals in randomly selected farms in LCC is described in the accompanying paper. Among animals from farms where no animals with clinical signs of FMD were present, the overall average percentage of positive swabs was above 10% while the percentage of positive swabs in farms with other animals having signs of healing/healed FMD was almost 50% and more than 80% of swabs were positive in farms with other animals having signs of acute FMD.

Figure 1 displays the FMDV infection prevalence at aggregate level from April 2006 to April 2007 among animals from farms where no clinical signs of FMD were present, based on the number of FMDV RNA positive animals found in a two-stage sampling scheme. The farm-level (herd-level) prevalence reflects the number of farms with positive animals, calculated as the proportion of farms with infected animals per month, and the animal-level prevalence reflects the number of FMDV RNA positive animals within the sampled population. Over the one-year period the mean animal-level prevalence for farms with PCR-positive animals was nearly 20% and the mean farm level prevalence was around 35% meaning that around one third of the 134 visited farms over the 13 months study period had an average of one in five animals positive for FMDV RNA.

The prevalence of FMDV RNA positive animals was very high in August 2006 where samples from all 9 sampled farms were positive for FMDV RNA (farm-level prevalence of 100%) and 17 of 27 individual samples were positive (animal level prevalence around 60%) possibly due to the rainy season in July and August. The moving average analysis (see Figure 1), which removes random variations within the point estimates, showed an appreciable increase in both farm-level and animal level prevalence from December 2006 to March 2007 (farm-level prevalence approaching 100% and animal-level prevalence at 50% or higher), likely expressing the cumulative effect of the second rainy season, the additional movements of animals caused by the Eid ul-Azaza festival (which that year fell on 31 December 2006) and possibly the slightly cooler temperature during this period (Figure 1).
3.2 Participatory information
During sampling the owners/managers of the farms were questioned about their FMD vaccine practice. Of the 127 farmers included in the questionnaire, 88% vaccinated their animals. Of those, 79% used trivalent Aftovax vaccine (Merial) and 9% the local monovalent type O vaccine. Another 4% mentioned that they did not know whether they had vaccinated against FMD, because they had vaccinated, but the vaccine used was unknown, while 8% did not vaccinate against FMD. All farmers that vaccinated their animals, administered the vaccine only once and not as recommended with an additional booster vaccination two to six weeks after the initial vaccination and moreover, only 4% were vaccinating their animals on a regular basis twice a year, whereas the majority of the farmers only vaccinated the new animals after they had arrived onto the farm.

In regard to the likely cause of new outbreaks of FMD on their farm, the owners/managers indicated that they believed that introduction of new animals was most important (43%) while introduction from the surroundings was rated as the second most likely cause (5%) while more than half of the replies indicated that they did not know (unknown/no reply 52%). Thus, if the unknown/no reply answers are not considered, around 90% of the farmers replying indicated that the most likely cause of new outbreaks was introduction of new animals while 10% considered introduction from the surroundings most likely.

3.3 Serology
The results of the antibody ELISA testing of the 180 samples collected (30 samples per month for 6 months) from the local slaughterhouse in LCC are shown in Figure 2 and Figure 3. The results indicated that the animals had a high level of antibodies against serotype O and serotype A during the whole period. Antibodies against serotype Asia 1 and serotype C (the latter likely due to vaccination or cross-reaction) were more variable although many animals were apparently positive. The levels of antibodies against these two serotypes were particularly variable, and low in several animals, in the November 2006 sampling. The reason for this is unknown, but it may correlate with the apparent low infection rate observed in LCC during the months of September, October and November of 2006 (Figure 1) although the circulating FMDV were clearly of serotype O and A (see later).

All 180 samples were also tested for antibodies against the non-structural proteins of FMDV and 176 were positive and only 4 samples negative (Figure 3). The calculated mean ODP for all 180 tested serum samples for antibodies to serotypes O, A and Asia 1 (at a 1:5 serum-dilution) were 9 ± 4, 6 ± 1 and, 8 ± 6, respectively, corresponding to an estimated endpoint titre of 1:320 and with a range of one twofold dilution step (i.e. endpoint titres of 1:160 to 1:640). For serotype C the mean ODP was significantly higher (20 ± 2), corresponding to an endpoint titre of 1:40 (range 1:20 to 1:80) and as stated above likely due to vaccination or possibly cross-reactions to the other serotypes.

To further substantiate these findings, 10 serum samples were randomly selected to determine the highest serum dilution that gave a positive signal in the ELISA for each serotype (Figure 4). The Median antibody titre for serotypes O, A and Asia 1 was 1:320 while the Median titre for serotype C was only 1:40. We also determined the endpoint titres of these 10 samples in the virus neutralisation test (Figure 5). Generally, the virus neutralisation titres were consistent with the results of the ELISA titration with the Median titres for serotypes O, A and Asia 1 equal to or above 1:100 with the titres for serotype A and Asia 1 being more variable than for type O. The Median virus neutralisation titre for antibodies against serotype C was less than 1:50 and with very little variability.

3.4 Sequencing and phylogenetic analysis
We sequenced the partial or full 1D coding region from the cDNA preparations from 58 of the 106 FMDV RNA positive swab-samples from animals without clinical signs (see accompanying paper). In addition, we sequenced the full 1D region from cDNA preparations from 17 epithelium samples collected during 2006. From all sequenced samples, 19 belonged to serotype O, including 9 swabs and 10 epithelium samples, and 56 to serotype A, including 49 swabs and 7 epithelium samples. Figure 6 shows the unrooted phylogenetic tree of the serotype O isolates from Pakistan in relation to similar serotype O sequences published in Genbank. The serotype O isolates from the Pakistan cluster are monophyletic and thus share a common ancestor. The most related isolates originate from Bhutan/Nepal, collected between 2003 and 2004 and belonging to a new Pan Asia lineage described by WRL at Pirbright in 2007 (http://www.wrlfmd.org) and designated Pan Asia II. Figure 7 shows a subtree of serotype O, containing only sequences from Pakistan, Bhutan, Nepal and Malaysia and demonstrates the close relationship between the isolates from Bhutan/Nepal and Pakistan. Noticing the small branch lengths, it is highly surprising that the sequence derived from the locally produced monovalent type O vaccine produced in Lahore is very closely related to samples derived from infected animals.
Figure 8 displays the deduced amino acid sequence of the partial VP1 sequence of the serotype O isolates. There is very high amino acid conservation, even though samples were collected from 2003 to 2006. However, the isolates from Pakistan are clearly distinct from the related isolates from Malaysia, Bhutan and Nepal at residues 143 and 200. Residue 143, located only 4 amino acids before the RGD motif in the GH-loop, contains histidine in the isolates from Pakistan whereas the others, like the majority of other published serotype O sequences, have proline at this position likely leading to a change in secondary structure.

Figure 9 shows the phylogram of the serotype A isolates. All the isolates from Pakistan belong to the recently discovered A/Iran/2005 lineage although the branch lengths, as typical for serotype A, are larger than those of serotype O.

### 3.5 Virulence and host species

In our study the majority of clinically affected animals infected with the FMDV A/Iran/2005 lineage were cattle (7 cattle and only 2 buffalo) while for FMDV type O, 13 out of 18 epithelium samples from clinically affected animals were from buffaloes and only 5 from cattle. In regard to the non-clinically affected animals, the results from the sequencing of FMDV RNA from 58 positive mouth swab samples showed that around 20% were positive for serotype O and the other approximately 80% positive for type A and approximately 90% came from buffalo and only around 10% from cattle. Taking these results together, and taking into consideration that the LCC has a large population of buffaloes and a minor population of cattle and that we took approximately 10 times more mouth swab samples from buffalo than from cattle, suggest that serotype O caused clinical FMD in both cattle and buffalo while in contrast, the A/Iran/2005 isolates described here, mainly caused clinical disease in cattle but subclinical infection in buffalo.

### 3.6 Sequencing and analysis of the complete coding sequence of selected A/IRN/2005 isolates and the potential for a recombination event

The complete coding sequence (CDS) of 3 A/IRN/2005-like isolates from epithelial samples collected in LCC (Pakistan1, Pakistan3 and Pakistan5) in early 2006 together with an isolate from the first recognized outbreak of the A/IRN/2005 lineage in Turkey in 2005 (sample kindly provided by WRL) were sequenced and compared with sequences available in Genbank. Phylogenies were then inferred for the complete CDS, as well as for each protein coding region of the FMDV genome. Figure 10 displays the phylogeny of the complete CDS of selected serotype O, Asia1 and A isolates and indicates a close relationship of the A/IRN/2005 subtype to the A22 and A28 subtypes circulating several years ago in the Middle East region. The A/IRN/2005 subtype appears to share a common ancestor with A Iran105 which may have originated in Iran in 1998. The relationships were further analysed by inferring phylogenies of the individual genome regions encoding for the structural proteins and subsequently the non-structural proteins. With the exception of IA (VP4) which is not surface exposed, distinct, serotype-specific clustering was observed in the structural region and the A/IRN/2005 sublineage obviously shares a common ancestor with A Iran105 from 1998 and is also related to the A22 lineage (Figure 11). However, when analysing the 1D genome region, encoding the VP1 protein, in more detail, the A/IRN/2005 sublineage clusters together with the A/IRN/99 sublineage while A Iran105 (A Iran105 not to be confused with the A/IRN/05 lineage) clusters together with the A/IRN/96 sublineage and the A22 sublineage is in this region well separated from the A/IRN/2005 sublineage (Figure 12). In contrast to the situation in the structural protein coding region, the non-structural protein coding regions displayed a much more complex phylogenetic picture. The phylogeny of the 2B region places the A/IRN/2005 sublineage in close relation to an A15 lineage from Thailand in 1960, A16 Belem from 1959, A12 Valle from 1932 and O5 India from 1962 while the inferred phylogeny of the 2C region indicates a clear relationship between the A/IRN/2005 sublineage and an Asia1 lineage from Lebanon, an Asia1 India97 vaccine strain as well as O1 Manisa. In both phylogenies non-serotype specific grouping can be observed between some Asia1, A and O sublineages, however the Pan Asia sublineage of serotype O and the A22, A23 and A28 sublineage of serotype A are monophyletic, i.e. each has apparently an inferred common ancestor well separated from A/IRN/2005 (Figure 13). The phylogeny of the coding sequence of the 3AB non-structural region indicated that the A/IRN/2005 sublineage related to a group of old serotype O, A and C isolates that are also related to the A/IRN/2005 sublineage in the 2B region while the phylogeny of the 3C protease region indicated that the A/IRN/2005 sublineage may share a common ancestor with the Pan Asia sublineage of serotype O and the Asia1 India97 vaccine strain. The 3C phylogeny indicated non-serotype specific clustering while the phylogeny of the 3D region indicated a potential relationship with the Pan Asia lineage of serotype O and with the previously mentioned group of old isolates and the A Iran105 and Asia1 India01 isolates. The phylogeny of the Leader protease region indicates that the A/IRN/2005 sublineage is related to the A22/A28 lineages, but still with a clear evolutionary distance (data not shown).
4. DISCUSSION

The results presented indicate that mouth swab samples collected into a suitable inactivating and stabilising buffer represent an easy way to ship samples to international laboratories and can be used to generate valid epidemiological information regarding the temporal prevalence of virus circulation and characterisation of circulating strains of FMDV by RT-PCR and sequencing. The apparent prevalence of subclinical infection in the LCC varied over the one year study period with low levels in April to July and September to November and with peaks in August 2006 and December 2006 to March 2007 coinciding with the periods with more precipitation and for the period stretching to March 2007 with the Eid ul-Azha festival that likely increases transmission due to the high number of animals transported across the country. The average percentage of FMDV RNA positive mouth swabs collected in randomly selected farms without clinical evidence of FMD was above 10% and assuming that these animals are infected and the detection window for FMDV RNA is around 14 days, then the accumulated "prevalence" of infection over a one-year period, the time period most animals stay in LCC before being transported for breeding or slaughter, would be between 200 and 300%, indicating that an individual animal may be exposed to circulating FMDV on average 2 to 3 times during a single lactation period in LCC. The rate of FMDV RNA positive animals was much higher in farms with clinical signs of healing/healed or with acute FMD where FMDV RNA positive mouth swabs reached 50% and 80% respectively, indicating that under the conditions observed in LCC all/most animals in an affected herd are exposed/infected to/with FMDV within a period of 3-4 weeks, however, more data is needed before any definitive conclusions can be drawn.

The indication of animals in LCC being exposed to FMDV on average 2 to 3 times a year corresponds well with our findings of significant circulation of FMDV serotype O and A together with other evidence pointing to the potential presence of serotype Asia 1 as reported by WRL in the period from 2002 to 2005. This significant circulation of FMDV is occurring despite widespread, but apparently non-effective, vaccination reported by the farmers, and although the effect of vaccination can not completely be ruled out, the serological results also support widespread circulation as indicated by 176 of 180 serum samples collected from the LCC slaughterhouse being positive for antibodies to FMDV non-structural proteins and with high titres of antibodies against serotype O and A. The titres of antibodies to serotype Asia 1 were also relatively high but more variable and this finding may be explained by a more limited circulation of this virus, consistent with us not finding any virus samples positive for type Asia 1, or with likely circulation of Asia 1 in previous years. The finding of low titres of antibodies to serotype C likely correlates with sporadic vaccination as the vaccines used by farmers are non-controlled, or could alternatively be due to cross-reactions caused by animals being infected and vaccinated multiple times taking into consideration that most animals slaughtered in LCC are at least 4 years of age and may have endured several lactation "cycles" in and out of the colony. Antibody titres against serotype Asia 1 and C were particularly variable and low in many animals sampled in November 2006 which may correlate to low circulation of FMDV in the preceding months and potentially supporting our hypothesis that at least part of the titres detected against Asia 1 and C is caused by cross-reacting antibodies generated by multiple infections and vaccinations together with recent infection with e.g. FMDV serotype O and A. However, at this point the exact explanation for this finding is currently unknown.

Sequencing of RNA extracted from 58 mouth swabs and 17 epithelium samples indicated widespread circulation of the Pan Asia II lineage of serotype O FMDV and the A/IRN/05 lineage of serotype A. Surprisingly, the sequence derived from the locally produced monovalent type O vaccine from Lahore was very closely related to the type O sequences from the field. The Lahore vaccine is reportedly based on a 30-year old vaccine strain which is clearly contradicted by the sequencing results; however, whether the vaccine has been recently updated is unknown. The RNA extracted from the vaccine was very difficult to RT-PCR amplify and sequence and we could thus only sequence a very small part of the genome and could therefore not determine whether the detected sequence has the hallmarks of cell culture propagated FMDV or alternatively may be a contaminant and not at all representative of the Lahore type O vaccine strain. Nevertheless, we consider the sequences derived from the field samples to be representative of the circulating strains which is further supported by the fact that although the type O sequences were related to sequences from isolates from Bhutan, Nepal and Malaysia, they had distinct differences, e.g. in the VP1 coding sequence. Additional sequencing of the complete coding sequence of 3 of the A/IRN/05 isolates from Pakistan suggested a complex evolutionary path for this sublineage involving recombination events possibly involving an A22-like ancestor for the structural protein region of the genome and an Asia 1-like ancestor for the non-structural protein region (Figure 14). This recombination event may have
occurred in the buffaloes which, in contrast to the situation for the circulating serotype O virus that apparently causes clinical FMD in both buffalo and cattle, appear to mainly become subclinically affected with the circulating serotype A virus possibly making concurrent infection with another FMDV, e.g. of serotype Asia 1, more feasible. This is currently only a working hypothesis, but nevertheless, it suggests that detailed phylogenetic analysis of the individual coding regions of FMDV may provide significant additional information not obtained by the more routinely used analysis of the VP1 region only.

Taking all the results into consideration, the vaccination practices in LCC should be significantly updated and enhanced in order to become effective. First of all, the vaccines used should be of high quality and contain the appropriate vaccine strains, currently including antigens covering the Pan Asia II lineage of serotype O, the A/IRN/05 lineage of serotype A and an appropriate antigen for serotype Asia 1. Moreover, the vaccinations will have to be applied in a more strategic way, if possible including vaccination of animals at origin once or better twice in the weeks before transportation into the colony occurs, combined with a twice annual mass vaccination scheme commencing in June and October-November before the rainy periods and thus providing herd immunity before the observed peaks in virus circulation. This advice may have to be modified to incorporate the fact that the timing of the Eid-ul-Azza festival, and the associated increased animal transportation across the country, moves forward a couple a weeks each year, and most likely one of the vaccination rounds should always occur prior to commencement of these animal movements.

5. CONCLUSIONS

- Sampling and testing of directly inactivated and stabilised mouth swabs, in combination with epithelial samples that may possibly also be inactivated and stabilised before shipment, have shown feasibility and strength in combined field and molecular epidemiological studies.
- The prevalence of circulating FMDV in LCC varied over the one-year study period and showed peaks associated with the rainy periods and the Eid-ul-Azza festival. Sequencing revealed circulation of the Pan Asia II lineage of serotype O and the so-called A Iran 2005 (A/IRN/05) lineage.
- The circulating serotype O FMDV apparently caused clinical FMD in both cattle and buffalo while the circulating serotype A FMDV mainly caused clinical FMD in cattle and subclinical infections in buffalo.
- Detailed analysis of the coding region of the A/IRN/05 FMDV indicated that this lineage may have evolved by recombination of a type A and a type Asia 1 FMDV, potentially by concurrent dual infection of buffaloes, possibly facilitated by the relative modest or absent clinical manifestations, but relatively high rate of subclinical infection, observed in buffalo.

6. RECOMMENDATIONS

- Collaborative studies using inactivated and stabilised samples and involving the relevant authorities and laboratories of countries with endemic FMD, together with International Organisations such as FAO, the WRL for FMD and a National Reference Laboratory from a resource-rich country may provide an efficient avenue for strengthening FMD control programs.
- Further studies should be supported to further establish the suitability of the proposed methods, to provide more epidemiological data and knowledge of circulating strains of FMDV in various settings and to establish the potential for using such samples to re-generate infectious FMDV by optimising the methods used for lysis and stabilisation, RNA extraction and in particular the methods for maximising the efficiency of transfecting susceptible cells cultures.
- An improved vaccination strategy for the LCC is proposed and if provided with international support, such a strategy could be established as a starting point for improved control of FMD in this setting. This would in turn provide improved outcomes for the many farmers and workers in LCC and in addition potentially provide a success story on which to base improved FMD control in other regions of Pakistan as well as in other countries with endemic FMD.

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8. REFERENCES


Figure 2
Descriptive statistics of the antibody ELISA for samples per month and serotype. Box-and-whisker diagram of the measured optical density percent (ODP) per month and serotype, showing the smallest observation, lower quartile (Q1), median, upper quartile (Q3), and largest observation. In addition, outliers according their interquartile range (IQR) and means are displayed. Each circle represents the measured ODP of a sample. The red line represents the threshold for each serotype, i.e., samples are considered negative if the ODP is for O > 25, A > 45, for Asia I > 35 and for C > 35.

Figure 3
Descriptive statistics of the ELISA results for all samples at a serum dilution of 1/5. Box-and-whisker diagram of the measured optical density percent (ODP), showing the smallest observation, lower quartile (Q1), median, upper quartile (Q3), and largest observation. In addition, outliers according their interquartile range (IQR) are displayed. Each circle represents the measured ODP of one sample.
Figure 4
Descriptive statistics of the antibody ELISA for 10 randomly selected samples per serum-dilution and serotype. Box-and-whisker diagram of the measured optical density percent (ODP) per dilution and serotype. Showing the smallest observation, lower quartile (Q1), median, upper quartile (Q3), and largest observation. In addition, outliers according to the interquartile range (IQR) and means are displayed. The top and bottom diamond vertices are the respective upper and lower 95% confidence limits (CI) about the group mean. Each circle represents the measured ODP of a sample. The red line represents the threshold for each serotype, i.e., samples are considered negative if the ODP is for O >> 50, for A >> 45, for Asia 1 >> 35 and for C >> 25.

Figure 5
Descriptive statistics of the virus neutralization test for 10 randomly selected samples per serotype. Box-and-whisker diagram of the calculated titer for each serotype. Showing the smallest observation, lower quartile (Q1), median, upper quartile (Q3), and largest observation. In addition, outliers according to the interquartile range (IQR) and means are displayed. The top and bottom diamond vertices are the respective upper and lower 95% confidence limits (CI) about the group mean. Each circle represents the calculated titer of a sample.
Figure 6
Unrooted phylogenetic tree of the partial 1D (VP1) nucleotide sequence of Pakistani serotype O isolates and related published sequences.

Figure 7
Bayesian phylogenetic analysis of the full 1D (VP1) nucleotide sequence of Pakistani serotype O isolates (black) and closely related published sequences (grey). The local produced monoventral vaccine (Lahore vaccine) is indicated in red.
Figure 5
Deduced amino acid sequence of the partial VP1 sequence of the genotype O isolates and related sequences from Malaysia, Pakistan and Nepal.

Figure 6
Bayesian phylogenetic analysis of the partial VP1 nucleotide sequence of Pakistani genotype A isolates (black) and closely related published sequences (gray).
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Figure 10 - Bayesian phylogenetic analysis of the complete coding sequence of the A/IRN/2005 sublineage (red) and related published sequences (black). Numbers on the nodes indicate clade credibility values.
Figure 11
Figure 12. Bayesian phylogenetic analysis of the 1D genome region of the A/IRN/2005 sublineage (red) and related published sequences (black). Numbers on the nodes indicate clade credibility values.
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Figure 13. Bayesian phylogenetic analysis of the genome regions 2B and 2C coding for non-structural proteins of the A/IRN/2005 sublineage (red) and related published sequences (black).

The New A/IRN/2005 sublineage
Recombination by concurrent dual infection with a type A and a type Asia 1 FMD virus???

5'UTR L 1A 1B 1C 1D 2A 2B CDS 3'UTR

3Dpolypomerase

VPs

NSPs

A22 ancestor??? Asia 1 ancestor???

Figure 14. The structural proteins region may have had a starting point in the A22 lineage with advantageous mutations leading to the occurrence of the A/IRN/2005 lineage.
Concurrently, recombination within the non-structural proteins region, potentially altering the virulence of the virus, may be involved in the success of this new sublineage.
The possible origin of this recombinant virus may be a co-infection with Asia1 and a type A precursor, possibly within the Asian Buffalos.